Antioxidant Activity of Vitamin E Determined in a Phospholipid Membrane by Product Studies: Avoiding Chain Transfer Reactions by Vitamin E Radicals

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Received February 24, 1997

Peroxidation of lipid membranes is implicated in a variety of damaging pathological events.[1] Consequently the antioxidant action of α-tocopherol (vitamin E), the principal natural lipid-soluble antioxidant in blood,[2] continues to be of major interest. The antioxidant activities of synthetic phenols as well as those of the vitamin E class are well-documented and interpreted for results in homogeneous solution in organic solvents.[3] Such determinations made in media which mimic natural biomembranes, such as micelles,[4] and especially in phospholipid membranes[5] are more difficult to determine reliably and to interpret satisfactorily. In particular these heterogeneous aqueous media appear to reduce the peroxyl radical trapping activity, k_{inh} of eq 1, of vitamin E and analogs by several orders of

$$\mathrm{ArOH} + \mathrm{LOO}^{\bullet} \xrightarrow{k_{inh}} \mathrm{ArO}^{\bullet} + \mathrm{LOOH} \qquad (1)$$

magnitude.[4,5] Such reductions of antioxidant activities in aqueous systems were attributed earlier to hydrogen bonding effects of the aqueous phase acting on the chromanol system.[4−6] However, both hydrogen bonding and limiting diffusion between the antioxidant and peroxyl radicals, especially for vitamin E in these systems, are factors to consider.[7] Indeed, it was concluded recently that only a "...small fraction of the observed rate diminution can be attributed to hydrogen bonding of α-tocopherol to water...".[7a]

Quantitative determinations of the inhibition rate constants, k_{inh}, have frequently used the inhibited oxygen uptake (IOU) method, which requires quantitative measurements of the oxygen consumed by the substrate during the induction period.[3−6] This method is subject to complications because of the chain transfer reactions arising[8] from reaction of the α-tocopheroxyl radical ($ArO^{\bullet}$) with hydroperoxides formed and with the unsaturated lipid to continue oxidation chains and regenerate ArOH. This regeneration of the ArOH radical could cause large effects in the measurements of antioxidant activities by the IOU method. These considerations and the unusual behavior of α-tocopherol in lipoproteic particles, such as LDL,[9] indicate the need for an independent determination of the antioxidant activity of α-tocopherol in membranes.

We now report on a quantitative determination of the inhibition rate constant for vitamin E in a phospholipid membrane based upon product studies which avoids complications due to chain transfer reactions of antioxidant radicals. This method makes use of the known direct relationship between the ratio of kinetic to thermodynamic peroxidation products formed and the hydrogen atom-donating ability of the medium, e.g., the concentration of the reactive unsaturated lipid in the uninhibited peroxidation and of the antioxidant in the inhibited peroxidation.[10] This method requires two straightforward product studies. The first one determines the *cis/trans* to *trans/trans* (*c,t*/*t,t*) ratio of hydroperoxides formed by oxidation at the 9- and 13-positions of the linoleate (18/2) chain as the membrane concentration of linoleate (LH) is varied. This is expected to follow a linear relationship as shown in eq 2,[10]

$$\frac{c,t}{t,t} = \frac{k_p}{k_\beta^{\mathrm{II}}(1-\alpha)}[\mathrm{LH}] + \frac{\alpha k_\beta^{\mathrm{III}}}{(1-\alpha)k_\beta^{\mathrm{II}}} \qquad (2)$$

where k_p is the rate constant for chain propagation of the lipid membrane, k_β^{II} and k_β^{III} are the rate constants for β scission of the *c,t*- and *t,t*-peroxyl radicals and $1 - \alpha$ represents the partitioning of the carbon radical between *c,t*- and *t,t*-peroxyl radicals.[10]

A second determination is made of the *c,t*/*t,t* ratio in the presence of known amounts of the antioxidant ArOH whose k_{inh} is to be measured. In the presence of the antioxidant, eq 3

$$\frac{c,t}{t,t} = \frac{k_{inh}}{k_\beta^{\mathrm{II}}(1-\alpha)}[\mathrm{ArOH}] + \frac{k_p}{k_\beta^{\mathrm{II}}(1-\alpha)}[\mathrm{LH}] + \frac{\alpha k_\beta^{\mathrm{III}}}{k_\beta^{\mathrm{II}}(1-\alpha)} \qquad (3)$$

controls the product ratio since now there are two H-donors in the medium—the lipid and the antioxidant, ArOH. To apply eq 3, it is necessary to ensure that both the lipid and the antioxidant remain constant during the experiment. Typically less than 5% of the lipid is oxidized in our experiment. We added an excess of ascorbate and homocysteine to the medium to keep the antioxidant in the reduced form. Ascorbate is known to regenerate vitamin E,[11] and homocysteine regenerates ascor-

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S0002-7863(97)00585-4 CCC: $14.00

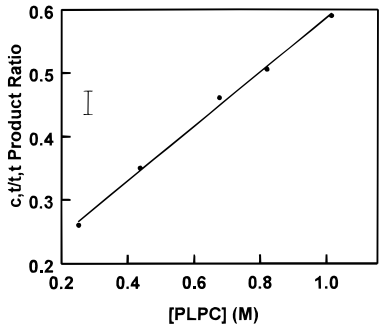

Figure 1. *cis*/*trans* to *trans*/*trans* (*c*,*t*/*t*,*t*) ratios of geometric isomers formed at the 9- and 13-positions of the linoleate chain versus the concentration of PLPC in the total lipid volume. The amounts of PLPC ranged from 5.44×10^{-6} to 4.69×10^{-5} mol. The amounts of DMPC ranged from 0 to 1.91×10^{-5} mol. Total lipid volume is calculated as the sum of the lipid volume of PLPC and the lipid volume of DMPC, in liters. Peroxidations were initiated by DBHN, 1.0×10^{-6} mol, at 37 °C in phosphate buffer, pH 7. The error bar, I, indicates average errors.

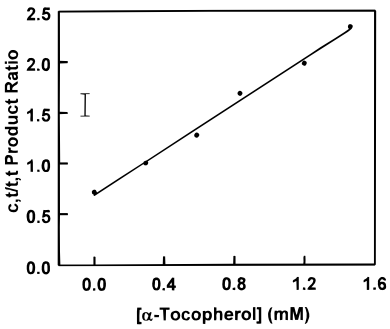

Figure 2. *cis*/*trans* to *trans*/*trans* (*c*,*t*/*t*,*t*) ratios of geometric isomers formed at the 9- and 13-positions of linoleate for various α-tocopherol concentrations in the PLPC lipid volume. The amounts of α-tocopherol ranged from 0 to 4.47×10^{-8} mol. The amounts of PLPC ranged from 2.98×10^{-5} to 3.39×10^{-5} mol. Peroxidations were initiated by DBHN, 1.0×10^{-6} mol, at 37 °C in phosphate buffer, pH 7. The error bar, I, indicates average errors.

bate.[12] Although the concentration of ascorbate and homocysteine will not remain constant during the run, this does not affect the results significantly since both ascorbate and homocysteine in the aqueous phase are only weak H-donors to peroxyl radicals formed in the lipid phase, under the present experimental conditions.[13]

The first series of experiments involved product studies from oxidation initiated by di-*tert*-butyl hyponitrite (DBHN) of 1-palmitoyl-2-linoleoylphosphatidylcholine (PLPC) over a 5-fold concentration in 1,2-dimyristoylphosphatidylcholine (DMPC) bilayers.[14] The required linear plot of these results (Figure 1) gives a value from the slope for the ratio $k_p/k_\beta^{II}(1 - \alpha) = 0.41$ M^{-1}.

The second series of experiments[14] involved oxidation of PLPC in the presence of vitamin E and excess ascorbate and homocysteine. The linear plot obtained over a 5-fold concentration of vitamin E is shown in Figure 2. The slope provides a value for the ratio of k_{inh}/k_β^{II} $(1 - \alpha) = 1.15 \times 10^3$ M^{-1} (eq 3). The ratio of the slopes from eqs 3 and 2 gives a measure of the antioxidant activity of α-tocopherol in terms of the ratio of rate constants, $k_{inh}/k_p = 2.81 \times 10^3$. This ratio is significantly higher than the value of 1.61×10^2 found in DLPC bilayers earlier by the IOU method[5] and about 6 times that of the ratio k_{inh}/k_p observed for the α-tocopherol-inhibited peroxidation of linoleate in micelles at 30 °C.[4c] We have recently determined the propagation rate constant, k_p, in PLPC bilayers to be 16.6 M^{-1} s^{-1} at 37 °C.[15] This provides a calculated k_{inh} for α-tocopherol in these bilayers of 4.7×10^4 M^{-1} s^{-1}.[16] This value is nearly an order of magnitude higher than that found in DLPC bilayers by the IOU method and should better reflect the antioxidant activity of vitamin E in biological and biomimetic systems.

Acknowledgment. This research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada, which is gratefully acknowledged. Fernando Antunes acknowledges a grant, BD/5575/95, from PRAXIS XXI/JNICT.

JA9705856

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(13) We find that the amounts of ascorbate (1.55 μmol) and homocysteine (2.34 μmol) in the absence of vitamin E gave *c*,*t*/*t*,*t* ratios of 0.70 ± 0.04, only marginally higher than from peroxidation of PLPC in the absence of any inhibitors, when *c*,*t*/*t*,*t* = 0.57 ± 0.01.

(14) The procedure for preparing these mixtures was used as in Barclay, L. R. C.; Baskin, K. A.; Kong, D.; Locke, S. J. *Can. J. Chem.* **1987**, *65*, 2541−2550. The linoleate oxidation products were analyzed as the corresponding hydroxy methyl esters. Samples were removed after less than 5% oxidation, immediately reduced by triphenylphosphine, and transmethylated followed by HPLC analysis using known procedures; see: Weenen, H.; Porter, N. A. *J. Am. Chem. Soc.* **1982,** *104*, 5216−5221. Barclay, L. R. C.; Artz, J. D.; Mowat, J. J. *Biochim. Biophys. Acta* **1995**, *1237*, 77−85. The *c*,*t*/*t*,*t* product ratios were the same after 2 or 3 h into a run, within experimental error, indicating that nonradical epimerization is not a significant factor.

(15) The propagation rate constant for PLPC was determined using the rotating sector method[4c] and modifications to the calculations which provide for corrections due to the dark rate and first-order termination. This method will be reported separately. The authors are aware of the uncertainty in the determination of k_{inh} by using a k_p from separate measurements, especially when using the rotating sector method on heterogeneous dispersions. Large errors may occur, even when this method is applied to homogeneous solutions; see: Howard, J. A.; Ingold, K. U. *Can. J. Chem.* **1965**, *43*, 2729−2736.

(16) This result was obtained from analysis of the data resulting from a 2-h reaction time. After 3 h of oxidation, a $k_{inh} = 4.8 \times 10^4$ M^{-1} s^{-1} was obtained, in reasonable agreement. Analysis for α-tocopherol by HPLC methods[2] after this time of reaction showed that there was no loss of this antioxidant. An experiment carried out using the same amount of initiator and α-tocopherol, but without vitamin C or homocysteine, showed a 40% loss of α-tocopherol after 3 h of reaction.